

## Expression of cellular retinoic acid-binding protein I and II (CRABP I and II) in embryonic mouse hearts treated with retinoic acid

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Cellular retinoic acid binding proteins are considered to be involved in retinoic acid (RA) signaling pathways. Our aim was to compare the expression and localization of cellular retinoic acid binding proteins I and II (CRABP I and II) in embryonic mouse hearts during normal development and after a single teratogenic dose of RA. Techniques such as real-time PCR, RT-PCR, Western blots and immunostaining were employed to examine hearts from embryos at 9–17 dpc. RA treatment at 8.5dpc affects production of CRABP I and II in the heart in the 48-h period. Changes in expression of mRNA for retinaldehyde dehydrogenase II (*Raldh2*), *Crabp1* and *Crabp2* genes also occur within the same time window (i.e. 10–11dpc) after RA treatment. In the embryonic control heart these proteins are localized in groups of cells within the outflow tract (OT), and the atrioventricular endocardial cushions. A gradient of labeling is observed with CRABP II but not for CRABP I along the myocardium of the looped heart at 11 dpc; this gradient is abolished in hearts treated with RA, whereas an increase of RALDH2 staining has been observed at 10 dpc in RA-treated hearts. Some populations of endocardial endothelial cells were intensively stained with anti-CRABP II whereas CRABP I was negative in these structures. These results suggest that CRABP I and II are independently regulated during heart development, playing different roles in RA signaling, essential for early remodeling of the heart tube and alignment of the great arteries to their respective ventricles.

**Keywords:** CRABP I, CRABP II, retinoic acid, neural crest, embryonic mouse heart

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### INTRODUCTION

Cellular retinoic acid binding proteins (CRABPs) are proteins of approximately 16–17 kDa, which bind retinoic acid (RA) with high affinity (Ong *et al.*, 1994). They belong to the multigene family of intracellular lipid-binding proteins (ILBP) (see Bernlohr *et al.*, 1997 for a review). To this day two types of these proteins have been characterized: CRABP I (Ong *et al.*, 1994) and CRABP II (Giguère *et al.*, 1990). Both proteins are present in the embryo as well as in the adult, and their expression is time- and tissue-specific (Dekker *et al.*, 1994; Bucco *et al.*,

1996), therefore each of these proteins is likely to have an important physiological function.

Both CRABP I and II are present in the cytoplasmic compartment of various cells (Gaub *et al.*, 1998) and CRABP II is also found in the nucleus (Delva *et al.*, 1999). Due to this fact as well as their high affinity and specificity for RA (Dong *et al.*, 1999) CRABPs are believed to messenger RA signaling in the cell. Both proteins might contribute significantly to RA metabolism, but their biological role has not been fully elucidated. The levels of CRABPs in the embryo undergo dramatic changes during development. Expression of CRABP II in murine embryo seems to be more widespread and at a lower level than that of CRABP I (Leonard *et al.*, 1995), and each of these is expressed in a tissue- and organ-specific manner (Dekker *et al.*, 1994; Bucco *et al.*, 1996) suggesting different functions.

It has been demonstrated that upon binding retinoic acid CRABP I participates in RA catabolism by producing its inactive metabolites (Boylan & Gudas, 1992). Because of this CRABP I is thought to buffer the amount of free intercellular RA and alter its amount entering the nucleus (Maden *et al.*, 1988; Perez-Castro *et al.*, 1989). In this way CRABP I may prevent teratogenic effects of excessive RA levels on various tissues (Dingle *et al.*, 1972) or may accumulate RA in the cells protecting them from possible hypovitaminosis A (Lampron *et al.*, 1995). On the other hand, CRABP II might be involved in transporting RA to the nucleus and therefore take part in the activation of nuclear RA receptors and regulation of RA-dependent genes (McCaffery & Dräger, 2000; Ross *et al.*, 2000; Zhelyaznik *et al.*, 2003; Theodosiou *et al.*, 2010).

Furthermore, promoters of both CRABP genes contain RA-response elements (RARE), being themselves targets for action of retinoic acid (Aström *et al.*, 1991; Durand *et al.*, 1992; Kleinjan *et al.*, 1998). However, their regulation by RA seems to be tissue-specific (Aström *et al.*, 1991; Leonard *et al.*, 1995). For instance an exogenous teratogenic dose of RA causes an increase of mRNA level for CRABP II in various cell lines and tissues *in vitro* (Lyn & Giguère, 1994; Williams *et al.*, 2004), whereas mRNA for CRABP I remains unaffected (Lyn

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**Abbreviations:** BSA, bovine serum albumin; CNCCs, cardiac neural crest cells; CRABP, cellular retinoic acid-binding protein; dpc, days post coitus; ECs, endothelial cells; EECs, endocardial endothelial cells; NCCs, neural crest cells; OT, outflow tract; RA, retinoic acid; RALDH2, retinaldehyde dehydrogenase II; SHF, second heart field; TBS, Tris-buffered saline.

& Giguère, 1994). This may indicate that the two genes are regulated differently.

CRABPs are markers for neural crest cells (NCCs), their derivatives (Ruberte *et al.*, 1990; Maden *et al.*, 1991; Ilagan *et al.*, 2006), and areas of intense morphogenetic processes in various embryonic tissues (Perez-Castro *et al.*, 1989; Maden *et al.*, 1991; Ruberte *et al.*, 1992) that correspond to sites of endogenous RA synthesis (Berggren *et al.*, 1999; 2001; Zhelyaznik *et al.*, 2003). CRABPs are also localized in areas prone to RA-related teratogenesis (Kalter & Warkany, 1961; Perez-Castro *et al.*, 1989; Ruberte *et al.*, 1990; Maden *et al.*, 1990; Leonard *et al.*, 1995).

It has been known for a long time, that RA, the active metabolite of vitamin A, is a potent, ubiquitous morphogene, crucial for prenatal vertebrate development and that it may also act as a teratogenic agent under conditions of its deficiency (Wilson *et al.*, 1953) or excess (Lammer *et al.*, 1985; Bouman *et al.*, 1995; Yasui *et al.*, 1995; 1997; 1999; Nakajima *et al.*, 1996; 1997; Lee *et al.*, 1998). Among tissues prone to RA-related teratogenesis is the developing heart (Wilson *et al.*, 1953; Lammer *et al.*, 1985; Rosa *et al.*, 1986; Bouman *et al.*, 1995; Yasui *et al.*, 1995; 1997; 1999; Nakajima *et al.*, 1996; 1997; Lee *et al.*, 1998; Sinning, 1998). The subpopulation of cells belonging to the second heart field (SHF) is important for heart tube development. The second heart field requires precise levels of RA signaling in a narrow time-window between 7.75 dpc and 10.5 dpc (Lavine *et al.*, 2005; Sirbu *et al.*, 2008). SHF and its proper addition to the developing heart tube are essential for correct alignment of the great arteries and normal development of the outflow tract (OT) (Yelbuz *et al.*, 2002; Waldo *et al.*, 2005). Both insufficiency and excess of local RA lead to serious cardiac defects, mostly within the OT region, for instance double outlet right ventricle (DORV), transposition of the great arteries (TGA), persistent truncus arteriosus (PTA), tetralogy of Fallot and others. RA signaling to SHF cells is mediated by fibroblast growth factor 8 (FGF8), whose proper amount is tightly controlled in SHF cells by RA (Ryckebush *et al.*, 2008) and also by the cardiac neural crest cells (CNCCs) approaching the heart (Hutson *et al.*, 2006). It has been demonstrated that the onset of RA-related teratogenesis within the heart occurs at around 8 to 10 dpc (Wilson *et al.*, 1953), which is when the NCCs are approaching and invading the heart (Hildreth *et al.*, 2008). At that time (8 dpc) the cells of SHF are also added to the developing heart tube (Kelly *et al.*, 2001).

We hypothesized that the expression and location of CRABP I and II in the developing heart may reflect the timeframe and areas of RA activity. Therefore, in this study we aimed to examine the expression and location of CRABP I and II proteins in the mouse embryonic heart during developmental stages from 9 dpc to 17 dpc and to compare these attributes with the developing hearts derived from RA-treated embryos. We propose that a single dose of RA administered at the time when the SHF cells contribute to the developing heart tube (Kelly *et al.*, 2001) may alter the expression of CRABPs genes and location of CRABPs proteins. Additionally, one of our goals was to compare the expression of mRNAs for both proteins during normal heart development and at corresponding stages after RA treatment. To study the possible relationship between CRABP I, II and endogenous RA we analyzed the location of retinaldehyde dehydrogenase II (RALDH2) — the key RA-synthetic enzyme in hearts from control and RA-treated

embryonic mice at the time of the assumed RA-mediated effect on the developing mouse heart (10–12 dpc).

## MATERIALS AND METHODS

The study was performed according to the requirements of the 2nd Local Ethical Committee at the Medical University of Warsaw.

The study was performed on Balb/c mouse inbred strain and on F1 cross of B57BL/6 and CBA mouse inbred strains. Balb/c and F1 cross females were mated with males of their respective strain for two hours in the morning. Spontaneous ovulation and subsequent mating was indicated by the presence of vaginal plugs. Mice that had successfully mated were housed separately for the purpose of collecting embryos. The time of joining the males with the females was recorded and was counted as day “0” for all obtained pregnancies. The remaining females were mated on the following days until impregnation.

At 8.5 dpc half of the pregnant females were treated i.p. with *all-trans-retinoic acid* (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 100 µl of dimethylsulfoxide (Sigma-Aldrich), at a dose of 70 mg/kg body weight. Embryos obtained from females that were not treated in any way (control embryos) served as comparison *versus* those exposed to a teratogenic dose of RA. In a pilot study performed on DMSO-treated control mice we obtained the same results with the RT-PCR method for CRABP I and II as on control non-treated mice. Three or more hearts or embryos were collected from every day of development for each procedure.

Hearts from control and RA-treated embryos were collected: 1. for RT-PCR and real-time PCR in RNase-free conditions, immediately frozen and kept at –80 °C for further analysis; 2. for tissue paraffin sections fixed in 4% buffered formaldehyde, rinsed with distilled water, dehydrated in increasing concentrations of ethanol and processed for paraffin embedding; 3. for Western blot in aseptic conditions, and immediately frozen and kept at –20 °C for further investigation. For Western blots with anti-CRABP I and II antibodies brains from embryos of all stages were also collected and kept frozen for further analysis. For Western blot with anti-RALDH2 antibodies the eye and the testis of adult mouse were collected as positive control tissue and kept frozen until used.

**Reverse transcription-polymerase chain reaction and real-time PCR.** Total cellular RNA was extracted by Qiazol extraction using a Tissue Lyser (Qiagen GmbH, Hilden, Germany), according to the instructions provided by the producer. Synthesis of cDNA was performed on 0.5–1 µg of total RNA with oligo-dT primers for 1 h at 42 °C using RevertAid M-MuLV reverse transcriptase (Fermentas, Ontario, Canada) according to vendor's instruction.

PCR amplification with *Taq* polymerase was performed using the following protocol: 1 cycle 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min.

Real-time PCR was carried out using a StepOnePlus apparatus (Applied Biosystem, Foster City, CA, USA) in a mixture containing 7.5 µl SYBR Green PCR Master Mix (SYBR Green qPCR Kit, Sigma, Poznań, Poland), specific primers and 50 ng of cDNA in a total volume of 15 µl using the following cycling conditions: 1 cycle of 95 °C for 15 min, followed by 40 cycles of 95 °C for 45 s, 58 °C for 45 s, and 1 cycle of 72 °C for 45 s. Primers specific for *Crabp I* gene (5'-TTC GCC GGT ACC

TGG AAG ATG-3', 5'-CCC CCT CAA GAA GTG TCT GTG-3', for *Crabp* II gene (5'-CGT GGA TGG GAG ACC CTG TAA G-3' and 5'-CAG GCA GTT CTT GGA CCC GTA G-3'), and for *Raldh2* gene (5'-TCC ATT CAC CAC TCT TGC CTC A-3' and 5'-AGA AGG GAA GGA AGC CAG TGC T-3') were used. EF2 (elongation factor 2; 5'-GCG GTC AGC ACA ATG GCA TA-3' and 5'-GAC ATC ACC AAG GGT GTG CAG-3') was also amplified under the same conditions and was used as a housekeeping gene. PCR efficiency was determined for all three pairs of primers. All reactions were performed in duplicates. Controls containing SYBR Green PCR Master Mix and primers without sample cDNA emitted no fluorescence after 40 cycles. Relative quantification of gene expression was calculated based on the comparative  $C_T$  (threshold cycle value) method ( $\Delta C_T = C_T$  gene of interest -  $C_T$  housekeeping gene). Comparison of gene expression in different samples was performed based on the differences in  $\Delta C_T$  of individual samples ( $\Delta\Delta C_T$ ). Results obtained from four different experiments were used to determine the level of mRNA for examined proteins. All data are presented as means  $\pm$  standard error (S.E.M.) and analyzed with Student's *t*-test. Differences were accepted as statistically significant at  $P < 0.05$ .

**Western blot.** Embryonic hearts and embryonic brains, collected at different days of development, as well as adult eye and testis were homogenized in extraction buffer (Ripa; 10 mM Tris/HCl; 0.15 M NaCl, 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS), containing protease inhibitors (0.005% aprotinin and 0.01% phenylmethylsulfonyl fluoride (PMSF) at +4°C.

**Protein determination.** One  $\mu$ l of embryonic heart extract or extraction buffer (blank test) and 9  $\mu$ l of deionized water was placed in a flat-bottomed 96-well plate and 0.2 ml of BCA protein assay reagent (Pierce, Rockford, Illinois, 61105 USA) was added to each well. The plate was incubated at 37°C for 30 min. Protein concentration was determined spectrophotometrically at 550 nm (SLT Spectra Lab instruments, Crailsheim, Germany). For RALDH2 blots total protein in lysates was determined with BCA™ Protein Assay (Thermo Scientific, Rockford, IL, USA). Protein transfer efficiency was determined by staining membranes with 1% Ponceau S in 1% acetic acid. After staining membranes were washed with 5% acetic acid, photographed, destained with tap water and blocked with 5% non-fat milk. Densitometric analysis was made with GelWorks 1 D Intermediate (UVP, Upland, CA, USA).

**Electrophoresis and Western blotting.** Embryonic heart extracts (10  $\mu$ g of protein) mixed in sample buffer with  $\beta$ -mercaptoethanol were separated by SDS/polyacrylamide gel electrophoresis (SDS/PAGE; 12% acrylamide) according to Laemmli (1970). Separated proteins were transferred onto PVDF membrane (Invitrogen, Carlsbad, CA, USA) by semi-dry blotting at 25 V for 30 min, using a Trans-Blot SD apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was incubated overnight with blocking buffer PBS (phosphate-buffered saline), pH 7.4, containing 0.1% Tween and 2% non-fat milk. Then the membrane was incubated overnight with primary antibody. For CRABP I the primary antibody was obtained from Chemicon (Billerica, MA, USA; cat. No. MAB5484) or from Abcam (Cambridge, UK, cat. No. ab2816), whereas for CRABP II an antibody from Chemicon (cat. No. MAB5486) was used. Anti-RALDH2 antibody was a gift from Peter McCaffery and was used for immunohistochemical staining.

For Western blot anti-RALDH2 antibody was also obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA, ALDH1A2, cat. No. # sc-22591). Goat anti-mouse immunoglobulins (DAKO A/S DK-2600 Glostrup, Denmark) served as the secondary antibody. Antibody binding was demonstrated by an amplified alkaline phosphatase detection system (Bio-Rad Laboratories, Hercules, CA, USA).

Due to the low level of CRABP I protein detected with the use of a standard Western blotting method (see above), chemiluminescence detection was additionally employed to assess the expression of this protein. After protein isolation (described above) and protein concentration measurement with the Quick Start Bradford Dye Reagent (Bio-Rad Laboratories) all samples were run on 12% SDS/polyacrylamide gel and transferred to PVDF membranes. Next membranes were immunoblotted with primary antibody against mouse CRABP I (Abcam, ab2816). All blots were then washed and incubated with an HRP-conjugated secondary antibody. The protein was then detected by enzyme-linked chemiluminescence with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

Due to the low level of RALDH2 antigen in developing heart (the protein is present only in the proepicardium of 9 dpc hearts and in the epicardial layer of mesothelial cells of 10–13 dpc hearts) the gel for standard Western blotting method was loaded with 25  $\mu$ g of protein instead of 10  $\mu$ g.

**Immunohistochemistry.** Paraffin sections were serially cut and subsequently deparaffinized on a hot plate for 30 min, immersed for 3  $\times$  10 min in xylene, and finally for 3  $\times$  10 min in 96% ethanol. After rinsing with PBS, the sections were treated in a microwave oven for 2  $\times$  5 min in 0.1 M citrate buffer (pH 6.0) in order to retrieve antigenic determinants. After cooling the sections were incubated with mouse monoclonal antibodies: anti-CRABP I (diluted 1:500–1:1000 in TBS containing 1% BSA) (Chemicon; cat. No. MAB5484), or with anti-CRABP II (diluted as above 1:1000–1:1500) (Chemicon; cat. No. MAB5486) overnight, at +4°C, rinsed in PBS, and incubated with anti-mouse IgG peroxidase complex (Chemicon) for 30 min. The color reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich), the reaction was stopped in distilled water and sections were counterstained with hematoxylin.

For RALDH2 staining paraffin sections were deparaffinized, treated with citrate buffer as above, and incubated overnight with anti-RALDH2 antibodies (1:4000). Subsequently sections were rinsed with PBS, incubated with anti-rabbit IgG-biotin (DAKO) (1:300) for 30 min, rinsed again with PBS, and incubated with streptavidin-HRP complex (DAKO) (1:300). The color reaction was developed with DAB substrate.

To determine antibody specificity negative control stainings were performed on some sections without the addition of a primary antibody (Fig. S7, figures denoted S — see Supplementary Material at [www.actabp.pl](http://www.actabp.pl)).

## RESULTS

### Expression of mRNA for *Crabp1*, *Crabp2* and *Raldh2* genes

In order to detect the expression of mRNAs for CRABP I and CRABP II RT-PCR technique was employed. In both control and retinoic acid-treated embry-



os CRABP I and II mRNA was detectable from 9 dpc to 17 dpc (not shown).

The embryos were exposed to RA on 8.5 dpc, as this is the known moment of embryonic expression of *Crabp1* and *Crabp2* (Lyn & Giguère, 1994) as well as based on the fact that two significant developmental events namely SHF addition to the heart tube and interaction between CNCCs and the SHF cells occur at that time, which are potentially affected by RA dosage (Nakajima *et al.*, 1996; 1997; Yasui *et al.*, 1995; 1997; 1999). Real-time PCR was employed to study quantitatively the expression levels of *Crabp1*, *Crabp2* and *Raldh2* genes. Assessment of these levels was begun shortly after RA treatment, at 9 dpc (hearts from earlier stages were too small for tissue preparation), and lasted until 12 dpc when the effect of RA on the levels of mRNA for all three genes seemed largely diminished.

We compared the basal level of expression of the examined genes at each day starting from 9 dpc. A strong decrease of mRNA levels for CRABP I and II was detected relative to the levels at 9 dpc (Fig. 1). A decline in RALDH2 mRNA was observed at 10 dpc, but in the next days the expression increased almost to the level observed at 9 dpc (Fig. 1).

The effect of RA treatment, on the mRNA levels, was similar for all three studied genes (*Crabp I*, *Crabp II* and *Raldh2*) (Fig. 2). In samples obtained from 9 dpc RA-treated embryos significantly lower levels of mRNAs for all three genes were detected compared with their control counterparts. However, in hearts from embryos collected at 10 dpc a reverse pattern could be observed, as mRNA production seemed to be greatly enhanced by the earlier RA treatment. At this developmental stage such difference in mRNA levels was much more apparent for both CRABP genes than for RALDH2. Similar, although slightly less pronounced up-regulation of all the genes studied, persisted in hearts of RA-treated embryos col-

lected on the following day (11 dpc). On 12 dpc mRNA expression for all three genes was similar in control and RA-treated hearts.

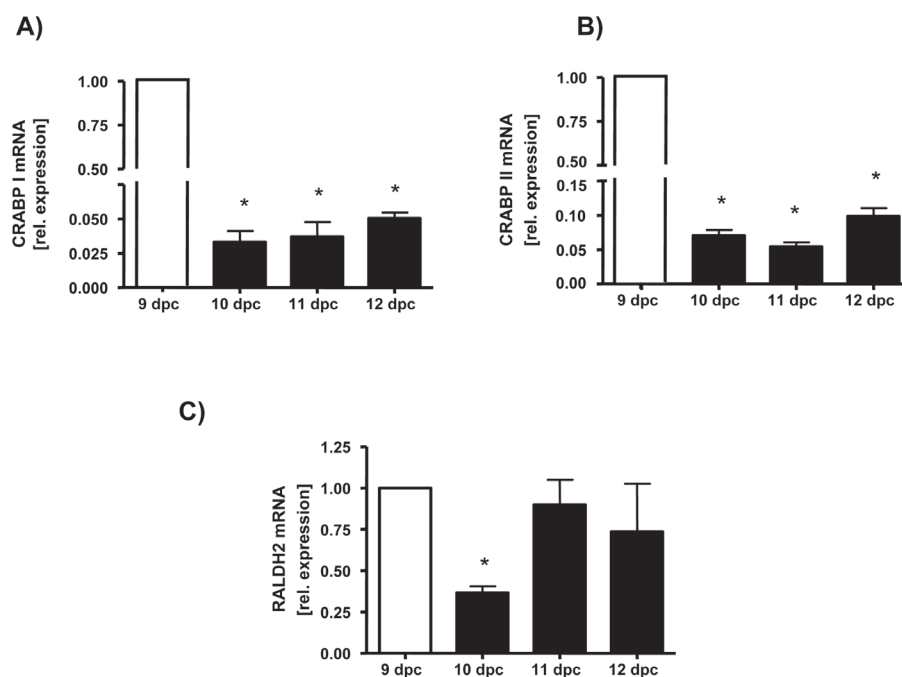
### Expression at the protein level

The Western blot was employed to determine whether an effect of RA on *Crabp1*, *Crabp2* and *Raldh2* genes similar to that found for mRNA could also be detected at the protein level.

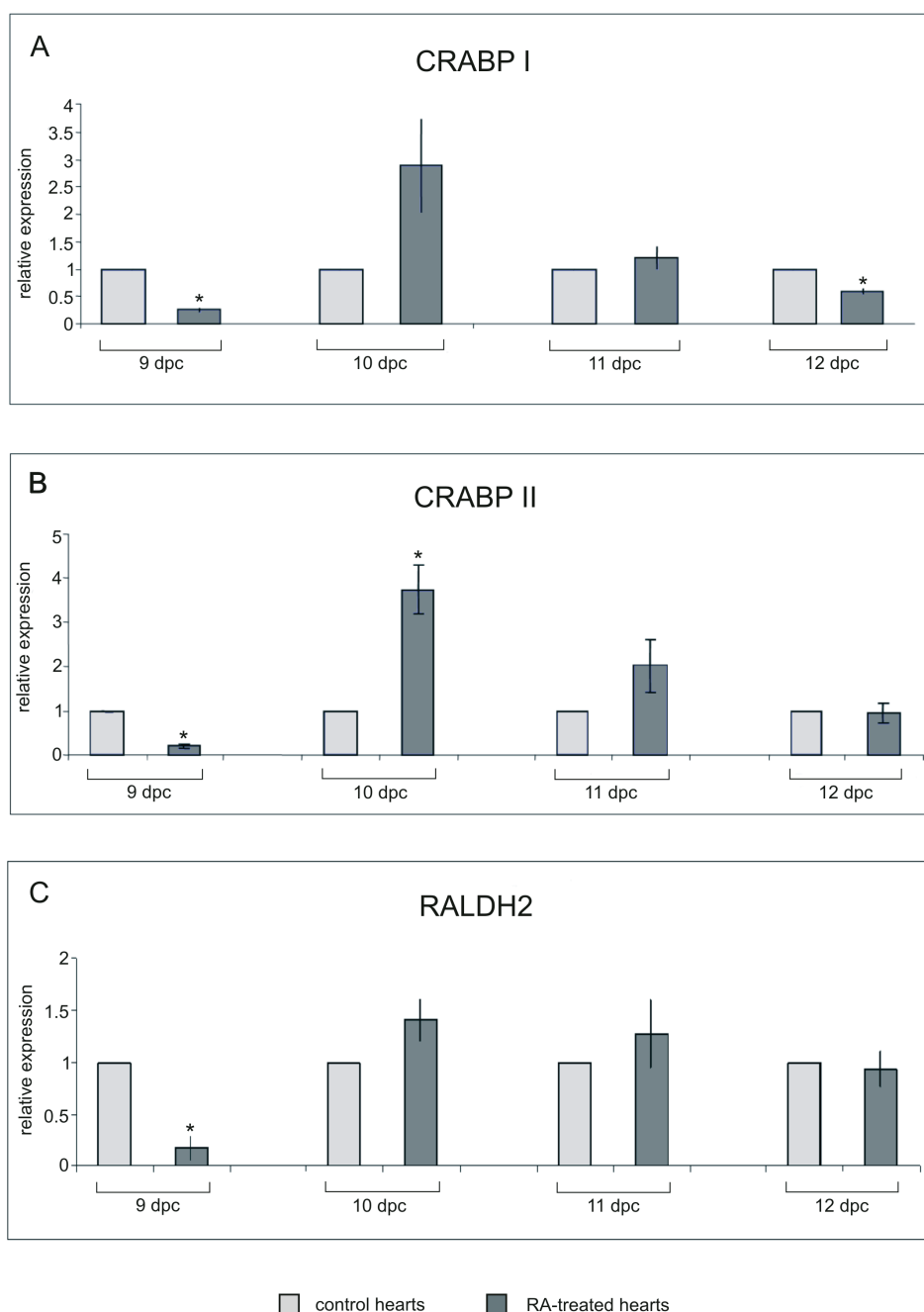
The bands obtained for CRABP I and II corresponded to the molecular mass of around 15–17 kDa (Fig. 3). Despite the low levels of CRABP I protein, which remained barely detectable even when chemiluminescence detection was used, an increased amount of CRABP I protein could be observed in 9 dpc heart following RA-treatment as compared to the control heart (Figs. 3 and 4A). This low amount of CRABP I protein in control hearts did not allow a comparison of the protein levels between control and RA-treated hearts at other time points studied. The level of CRABP II protein at 9 dpc in the control heart was very low and high after RA treatment (Fig. 4B). It remained relatively high at 10 dpc in both control and RA-treated hearts (as compared to its level in 9 dpc control heart), and declined thereafter (Fig. 4B). The only distinctive response to RA treatment was detected at 9 dpc in the levels of both CRABP I and II proteins suggesting similar regulation of both proteins by RA.

The expression of RALDH2 protein seemed not to be changed by RA treatment at 9–12 dpc (Fig. 4C) compared to control hearts.

Despite the obvious initial effect of RA treatment, the results obtained for later days of development did not suggest any further major changes in the level any of the proteins both in the course of normal heart development or following RA treatment. Therefore we ceased to study the levels of expression of all three proteins after 12 dpc.



**Figure 1.** Real-time PCR for CRABP I (A), CRABP II (B) and RALDH2 (C) in control heart extracts from 9 dpc through 12 dpc. Temporal dynamics of mRNA expression was normalized to the level on 9 dpc. Each column represents the mean  $\pm$  S.E.M. of four independent experiments run in duplicate. Values marked with a star vary significantly ( $P < 0.05$ ) from the level on 9 dpc.



**Figure 2. Real-time PCR for CRABP I (A), CRABP II (B) and RALDH2 (C) in control and RA-treated heart extracts from 9 dpc through 12 dpc**

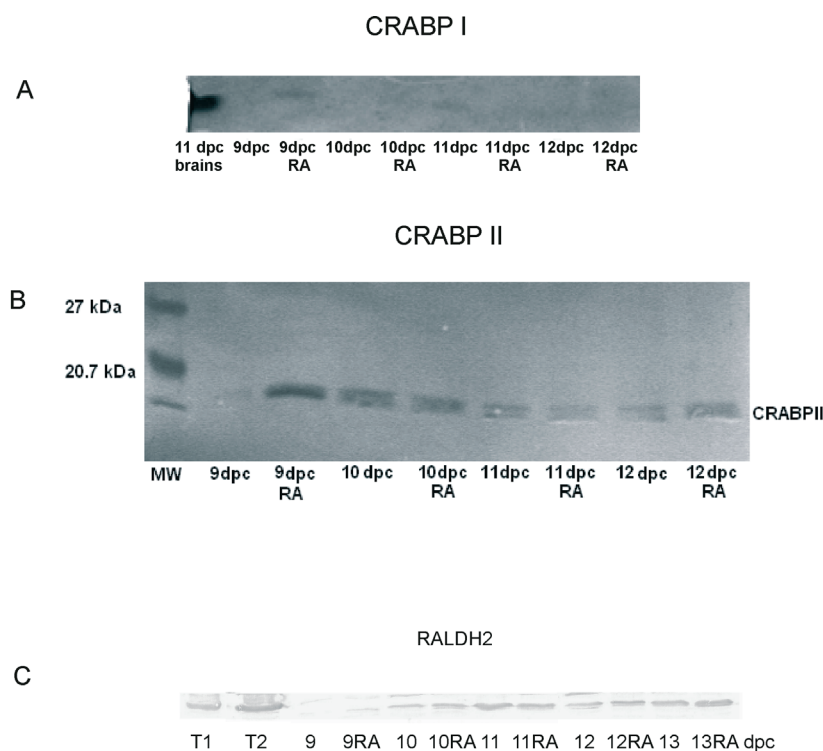
Comparison of mRNA levels for control and RA-treated hearts at each studied time point. Each column represents the mean  $\pm$  S.E.M. of four independent experiments run in duplicate and normalized to EF2 expression. At each time point, the value in control hearts is set as 1. For each time-point value in RA-treated hearts marked with a star varies significantly ( $P < 0.05$ ) from its control counterpart.

#### Localization of CRABP I, CRABP II and RALDH2 proteins

To compare the localization of CRABP I and II proteins in both control and RA-treated hearts immunohistochemistry was used. Although quantitative studies showed only a short-term response to RA treatment at both mRNA and protein levels, an immunohistochemical study was carried out until the last day of pregnancy (17 dpc) in order to determine whether a single teratogenic dose of RA induces any persistent spatial changes in the expression of CRABP I and II within specific cardiac structures.

At all the stages studied (Figs. S1–S6, and not shown), immunolabeling for CRABP I and II was detected within embryonic hearts. Both proteins were also present in hearts of RA-treated embryos. These results were validated with the use of negative controls (sections stained without the addition of a primary antibody) (Fig. S7) to eliminate false positives.

At the earliest stages, embryonic hearts exhibited fairly homogenous staining of different areas of the myocardium, which sometimes varied in intensity between CRABP I and II (10 dpc, Fig. S1, a and c, b and d; 12 dpc, Fig. S3, b and f, d and h). The only notable exception from this homogenous staining occurred at



**Figure 3. Western blot analysis of CRABP I (A), CRABP II (B) and RALDH2 (C) expression between 9 and 12 dpc in control and RA-treated heart extracts.**

The bands detected with anti-CRABP I (Abcam, ab2816) and anti-CRABP II (Chemicon, MAB5486) antibodies correspond to molecular mass of about 15–17 kDa, confirming antibody specificity. The band of 55 kDa corresponds to RALDH2 protein (C). For CRABP I detection control tissue (brain) was loaded in the first lane. For RALDH2 detection control tissue consisted of mouse testis homogenate loaded in the first lane at 25 µg (T1) and in the second lane at 50 µg (T2).

11 dpc in control hearts, in which CRABP II location exhibited a gradient of staining intensity along the myocardium (Fig. S2d, e), being the strongest in the OT and atria. By contrast, the staining for CRABP I was evenly distributed within the ventricle, the atrium and the OT (Fig. S2a, b), and no such labeling gradient could be detected in RA-treated hearts stained for CRABP I or II (Fig. S2c, f). Besides this distinctive staining pattern in the myocardium, it is also worth noting that at 12 dpc after RA treatment the staining for anti-CRABP I exhibited a patchy pattern in aggregations of cells distributed evenly within the compact layer of the ventricles, ventricular trabeculae, the atrial myocardium, and the scattered cells of the endocardial cushions (Fig. S3c and d). Higher magnification photographs revealed that this labeling was mostly located in the cytoplasm of the cells (Fig. S3, inset in d). Such labeling pattern resembling patches or dots could also be detected on some of the following days of development both in control (not shown) and RA-treated (Fig. S4c, d) hearts.

Additionally, a strong staining of endocardial endothelial cells (EECs) for CRABP II could be observed within the ventricles (Fig. S3b, f) and the atria (not shown) at 12 dpc as well as in EECs of the OT cushions, and the developing semilunar valves, great arteries (Fig. S4e, f, g, Fig. S5f, g) and atria (Fig. S5h) at 13–14 dpc. Moreover, the mesenchymal cap of the forming interatrial septum exhibited staining for CRABP II at 11 (not shown) and 12 dpc (Fig. S3e, white arrow).

On the following days of heart development (13, 14 dpc) homogenous labeling for CRABP I and II was de-

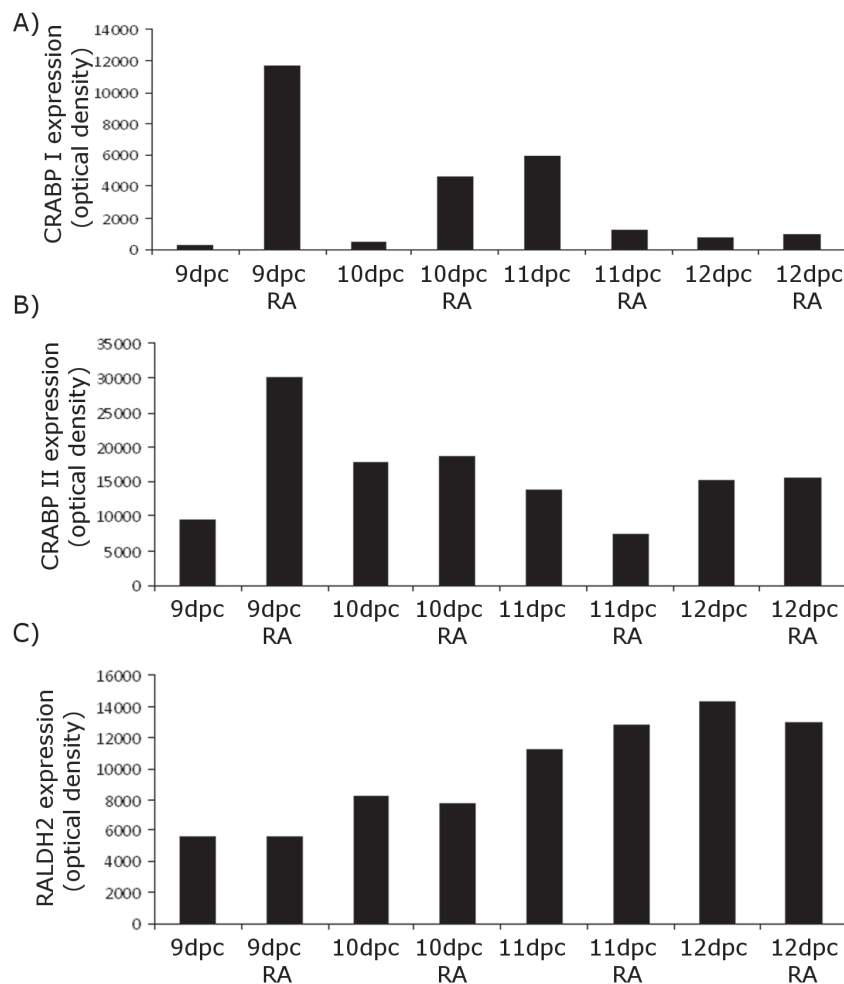
tected in the ventricles, atria and the great arteries of control hearts (Fig. S4a, b, e, f) and hearts from RA-treated embryos (Fig. S4c, d, g, h; Fig. S5a, b, e, f) except for 14 dpc when the walls of great arteries exhibited significantly diminished staining (Fig. S5a, b, e, f, g). Additionally, at 13 dpc and 14 dpc in both control and RA-treated hearts, some of the mesenchymal cells of the OT cushions (Fig. S4a, b, e, f) and the forming aorto-pulmonary septum (Fig. S5c, e, f, g) were labeled for CRABP I and II. The number of CRABP I and II positive cells in the OT cushion was, however, diminished in RA-treated hearts. As shown in insets in Fig. S4f and Fig. S5g the color reaction in these cells was mainly restricted to the cytoplasmic compartment.

At later stages of development (15–17 dpc) in control hearts as well as those from RA-treated embryos the staining for CRABP I and II was evenly distributed within the myocardium except for the wall of the great vessels and the semilunar valves which showed no staining for either protein (not shown). At these stages of development staining for CRABP I and II was detected in the mesenchymal cells of the aorto-pulmonary septum (not shown) in hearts from control and RA-treated embryos. Labeling for both proteins was also present in the mesenchyme of the atrioventricular valves, localized close to the surface adjacent to the ventricular lumen in the control hearts. Similar staining for CRABP I and II could be observed in the mesenchyme of the atrioventricular valves following RA administration.

Throughout the stages studied the epicardium remained weakly stained for both CRABP I and II in control and RA-treated hearts. Interestingly in 13-dpc hearts the mesenchymal tissue underneath the epicardial layer exhibited positive staining for CRABP I in RA-treated hearts (Fig. S4d).

Besides the heart, color reaction was also observed in other developing tissues of the studied embryos, most notably in the neural epithelium and neural ganglia, gut mesenchyme, somites, limb mesenchyme, mesonephros, gonads and tail mesenchyme, which is in accordance with previous studies (Perez-Castro *et al.*, 1989; Maden *et al.*, 1991; Ruberte *et al.*, 1992; Kleinjan *et al.*, 1997). Staining for CRABP II was absent from the developing hepatic, osseous and cartilaginous tissue at all time points of the study.

Additionally, immunohistochemical staining for retinaldehyde dehydrogenase II (RALDH2) was performed in 10–12 dpc hearts. This technique was employed to determine areas of endogenous RA synthesis within the control and RA-treated hearts at these early stages of development, corresponding to the stages when RA-mediated change of mRNA and protein levels for *Crabp1* and *Crabp2* genes was detected. RALDH2 was detected



**Figure 4. Optical density of Western blot for CRABP I (A), CRABP II (B) and RALDH2 (C) protein bands**

Ponceau S optical density was used as a correction factor for quantification.

in the forming epicardium of 10, 11 and 12-dpc hearts (Fig. S6a–f). Additionally, this enzyme was present in some subepicardial cells penetrating towards the myocardium of 12-dpc hearts (not shown). These scattered cells are probably derived from the epicardial layer. In the RA-treated hearts from 10 and 11 dpc embryos, RALDH2 staining of the epicardial tissue was markedly reduced (Fig. S6d, e) and at 10 dpc it was also present in the myocardium, most notably in the region of the OT (Fig. S6d). Exceptionally strong RALDH2 staining was also observed in the surrounding extracardiac tissues (Fig. S6d, white arrow). Additionally, RALDH2 labeling was detected in proepicardial vesicles of control (Fig. S6, b) and RA-treated (inset in Fig. S6e) 9, 10 and 11-dpc hearts and in the forming blood islands of RA-treated, 12-dpc heart (Fig. S6f).

## DISCUSSION

Although embryonic expression of CRABP I and II in the mouse has been described by several research groups (Ruberte *et al.*, 1991; 1992; Lyn & Giguère, 1994; Leonard *et al.*, 1995), the presence of these proteins in the developing heart has not been given much attention. The results of studies aimed to detect embryonic expression of CRABP proteins seem to be somewhat contradictory

in relation to this organ. While some authors describe cardiac expression of these proteins, albeit at low levels as compared to other embryonic structures (Perez-Castro *et al.*, 1989; Ruberte *et al.*, 1991; 1992), others failed to detect expression of these proteins in this developing organ (Kleinjan *et al.*, 1997). Maden (1994) demonstrated CRABP II in the heart whereas CRABP I was not detected. These inconsistencies may be due to sensitivity of particular methods used in those studies.

Therefore in the present study we focused specifically on embryonic mouse heart, aiming to describe the dynamics of CRABPs expression in this organ at a critical period of its development. We also wanted to study the expression of RALDH2, the major RA-synthesizing enzyme in the heart, and the influence of exogenous RA treatment on RALDH2 protein and mRNA expression.

In this study we have shown that CRABP I and II proteins and their genes are expressed in embryonic mouse hearts at 9–17 dpc. We have demonstrated that:

1. The level of mRNA for CRABP I and II varies among the developmental stages of normal hearts and hearts from embryos treated with a single exogenous dose of RA.

2. As compared to control samples, the level of mRNA for both CRABP I and II after RA treatment decreased at 9 dpc, and increased at 10 dpc. The same tendency was observed for mRNA of RALDH2.

3. RA treatment increased the expression of CRABP I and II protein in the heart within the first 24 h following treatment, with a subsequent decrease to a level found in control embryos. RA did not cause any variation in RALDH2 protein level within the first 24 h after treatment.

4. A staining “gradient” of CRABP II was observed in the arterial and venous poles of 11-dpc hearts, which may reflect the fact that RA mediates a developmental signal for these areas at that time. It is also possible that it is connected with the invasion of these structures by CNCCs. Both CRABP proteins were expressed in some cells within the OT. At the cellular level CRABP II was found transiently in some cells which remained negative for CRABP I throughout the study, for instance the endocardial endothelial cells.

The variation of mRNA levels for CRABP I and II within the 48 h after RA treatment as compared to control hearts suggests that this single dose of RA exerts its effect on the expression of both genes. A similar tendency was observed for the *Raldh2* gene. The increased level of CRABP I and II protein in embryonic hearts



within the first 24 h after RA treatment suggests that at the protein level CRABP I and II are also subject to RA regulation. This effect may be caused by an immediate up-regulation of mRNA for CRABP I and II, within a few hours after RA treatment, which would be consistent with previous observations on up-regulation of their genes in various cellular models *in vitro* (Boylan & Gudas, 1992; Lyn & Giguère, 1994; Williams *et al.*, 2004). After studying the effect of RA treatment on the expression of various neural crest genes, Williams *et al.* (2004) classified *Crabp* II into a group of genes characterized by an immediate alteration of their mRNA levels in response to RA. Such reaction to RA occurred mostly within 6 h after treatment, and ceased after 12–24 h. In our study both CRABP I and II mRNAs were down-regulated after 12 h following RA administration, that is at 9 dpc. In relation to these results, the increased protein level in 9-dpc RA-treated hearts observed in our study could be due to immediate gene upregulation by RA. In this scenario, an increased level of CRABP I and II protein could be observed even at 9 dpc, that is 12 h after RA dosage, when mRNA for these genes, initially up-regulated directly after RA dosage, had already been degraded. In addition a down-regulation of mRNA for CRABP I and II could be a result of negative feedback regulation of these genes by their products, that is by an excess of CRABP I and II proteins. At this time point (9 dpc) CNCCs, which are considered to be the major source of CRABP proteins in the developing organs, are not yet present in the heart (Hildreth *et al.*, 2008), therefore the abundance of CRABP I and II proteins following RA treatment is likely the result of RA-induced ectopic expression. Subsequent upregulation of mRNA for CRABP I and II proteins at 10 dpc may indicate that a single dose of RA given at 8.5 dpc may also exert a further, delayed effect on their expression. We presume that this secondary upregulation may become more complex, not solely dependent on RA action, but involving other regulatory factors, for instance a diminished amount of gene end-products.

It is worth noting that specific molecular factors involved in RA-driven regulation of *Crabp1* and *Crabp2* genes are not yet fully determined. A study by Wang *et al.* (2004) points to a likely role of BAF180 of a chromatin remodeling complex PBAF in coactivation of *Crabp2* gene presumably as a cofactor of nuclear RA receptors such as RXRa. Ablation of BAF180 causes cardiac defects characteristic for disrupted RA signaling pathway (Kastner *et al.*, 1994; Sucov *et al.*, 1994) such as hypoplastic ventricular myocardium and VSD (Wang *et al.*, 2004). BAF180 has also been characterized as a factor indispensable for *Crabp2* response to RA and this protein has been evidenced to bind directly to *Crabp2* promoter region (Wang *et al.*, 2004). An effect of BAF180 ablation on the level of *Crabp2* gene expression has also been described. It seems that BAF180 ablation and thus disruption of RA signaling pathway may cause either an increase or down-regulation (Wang *et al.*, 2004) of expression of this gene. Such discrepancies may be explained by the different effect of RA signaling on particular genes based on specific localization in the embryonic heart, as the effect of RA is thought to be highly cell- and tissue-specific, and may be different for the same gene depending on the site of its expression (Wang *et al.*, 2004, and references therein).

The fluctuations in the levels of mRNA and protein for CRABP I and II are the highest during the first 72 h following RA treatment. It is known that at this

stage of cardiac development, CNCCs reach the heart (Poelmann & Gittenberger-de Groot, 1999; Jiang *et al.*, 2000; Hildreth *et al.*, 2008) and the OT undergoes marked differentiation during which SHF cells are added to the developing OT and the proper alignment of the great arteries with their respective ventricles occurs (Kelly *et al.*, 2001; Mjaatvedt *et al.*, 2001; Yelbuz *et al.*, 2002; Waldo *et al.*, 2005; Buckingham *et al.*, 2005; Rochais *et al.*, 2009). At this time a “staining gradient” with anti-CRABP II was observed. This staining gradient for CRABP II, a marker of NCCs, was detected in the OT and the venous pole of 11-dpc control hearts. Firstly, such localization of staining may mark the cardiac areas populated by the NCCs, as both CRABP I and II are known to be molecular markers of these cells (Williams *et al.*, 2004; Ilagan *et al.*, 2006). This gradient, however, was not observed with anti-CRABP I antibodies, which may indicate that these two proteins are controlled differently. Additionally, this staining pattern may also indicate the sites of developmental action of RA signaling since CRABP II is thought to transduce the RA signal (McCaffery & Dräger, 2000; Ross *et al.*, 2000; Zhelyaznik *et al.*, 2003). These areas seem to converge with the fields of RA action within the heart which have been characterized by Moss *et al.* (1998), where both synthesis (sinus venosus, atria) and response to RA (sinus venosus, atria, and conotruncal area) is known to occur. SHF cells are themselves extremely sensitive to RA action during normal heart development, which has been demonstrated in mammals and other vertebrates (Collop *et al.*, 2006; Vermot *et al.*, 2006; Ryckebusch *et al.*, 2008; Sirbu *et al.*, 2008; Waxman *et al.*, 2008; Rochais *et al.*, 2009). Thus an excess of RA in our model causes heart malformations in the area where derivatives of the SHF are located and NCC cells accumulate (OT). Excessive RA signaling is known to disturb correct migration of the NCCs (Moss *et al.*, 1998; Niederreither *et al.*, 1999) and perturbs signaling between NCCs and SHF cells *via* action on Fgf8 (Hutson *et al.*, 2006; Ilagan *et al.*, 2006; Park *et al.*, 2006). Fgf2 and Fgf9 are two other retinoid-induced growth factors which regulate heart development (Lin *et al.*, 2010). It is interesting whether CRABP II participates in RA-dependent control of SHF and NCC cellular populations contributing to the arterial and the venous pole of the heart at that time. In that case CRABP II could be involved in sustaining locally-specific levels of RA signaling in these areas, which could be different from the levels of this signal in the ventricular compartment of the heart tube, based on the distinct origin of SHF and the NCCs. Exogenous, teratogenic doses of RA could affect this highly specific signaling and the expression of CRABP II as its component suppressing its gradient as seen at 11 dpc. Ectopic expression of RALDH2 in the outflow portion of the heart at 10 dpc could also point to a role of excess RA in disrupting the normal pattern of endogenous RA signaling.

However, the putative participation of CRABP II in mediating RA signal for the developing heart requires confirmation with more detailed studies. The question whether suppression of CRABP II staining gradient following RA treatment represents diminished contribution of the NCCs to the venous and arterial pole of the heart also remains open, as more molecular markers of the NCCs, preferably those not regulated by RA, would have to be used in order to resolve it.

At the intracellular level both proteins (CRABP I and II) were mostly located in the cytoplasmic compartments



of cardiac myocytes and other cell types at these stages of development.

Interestingly, groups of cells in the OT cushions and the atrioventricular cushions at 11–13 dpc expressed both proteins and the number of these cells diminished after RA treatment. Also the labeling intensity between CRABP I and CRABP II in these groups of cells varied. This would be consistent with previous studies describing embryonic contribution of NCCs to specific cardiac structures (Hildreth *et al.*, 2008), as well as reports claiming that such contribution can be diminished by RA treatment (Maden *et al.*, 1996; Hoover *et al.*, 2008). However, as stated above it is not clear whether such diminished staining can be directly identified with a lower contribution of the NCCs.

At 13–14 dpc a strong, distinct staining for CRABP II was also detected in the endocardial endothelial cells (EECs) of the OT, in the EECs of the right ventricular infundibulum, atria, and the forming semilunar valves in both control and RA-treated hearts. In contrast, CRABP I was absent from the EECs, suggesting that the roles played by both proteins in RA metabolism in various structures of the developing heart are different. Such a strong labeling for CRABP II might be explained by its upregulation by endogenous RA, as, according to the literature, the RA signal for cardiomyocyte development may come from the epicardium as well as from the endocardium (Kang & Sucov, 2005). The developmental signal for nonmyogenic structures of the heart, such as the aorta and pulmonary arteries, semilunar valves and atrioventricular valves, which have been classified into one of cardiac “fields” of RA morphogenetic action (Moss *et al.*, 1998), may be derived from the endocardium, initiated by an RA-synthesizing enzyme other than RALDH2 (as suggested in: Moss *et al.*, 1998) and mediated specifically by CRABP II. On the other hand, RA signaling for the atrial and ventricular myocardium seems to be generated in the epicardium by RALDH2 and further mediated by CRABP proteins present in the ventricular and atrial myocytes. The presence of RALDH2 enzyme detected within the epicardium in this study as well as in previous publications (Moss *et al.*, 1998; Berggren *et al.*, 1999; Xavier-Neto *et al.*, 2000; Berggren *et al.*, 2001; Hoover *et al.*, 2008) combined with the presence of cells stained positively for RALDH2, which migrate from the epicardium towards myocardium, could also support these claims.

RALDH2 has been characterized as the major RA synthesizing enzyme in the embryo (Zhao *et al.*, 1996; Berggren *et al.*, 1999; Vermot *et al.*, 2006). We were interested whether exogenous RA would alter the level of *Raldh2* gene expression. We observed a down-regulation of *Raldh2* mRNA in RA-treated hearts at 9 dpc, but at 10 and 11 dpc this gene was up-regulated. Susceptibility of this enzyme to regulation by its own product has been proven in P19 embryonal carcinoma cells (Zhao *et al.*, 1996). Similarly Napoli (1999) classifies *Raldh2* as an RA-responsive gene, noticing that its response to RA might be tissue specific. A response of RALDH2 to RA has also been detected in some structures of the murine embryo, 12 and 27 h after maternal RA treatment, which was administered precisely at 8.5 dpc (Neiderreither *et al.*, 1997b). Regardless of the exact mechanisms responsible for the fact that the reaction of RALDH2 to exogenous RA varied in time, these results clearly show that a single dose of retinoic acid administered at the critical time of cardiogenesis (8.5 dpc) may affect production of endogenous RA within the developing heart.

Furthermore, immunohistochemical staining with anti-RALDH2 antibodies following RA treatment reveals additional signal, although weak, in the myocardium of the OT and the ventricles at 10 dpc. This ectopic expression of RALDH2 does not, however, correlate with the level of total RALDH2 protein expression at that time (Fig. 4C).

A strong distinctive pattern of RALDH2 labeling is also present in the distal portion of the outflow tract as well as in surrounding tissues. These areas are known to be in spatial contact with the NCCs immediately before the immigration of these cells into the outflow tract. It is thought that RALDH2 is not expressed in the migrating CNCCs (Niederreither *et al.*, 1997a; Berggren *et al.*, 1999). However, it is believed that for successful migration of these cells, a specific pattern of RA must be provided throughout their migratory pathways (Niederreither *et al.*, 1997a; Berggren *et al.*, 1999). It is therefore possible that aberrant induction of RALDH2 expression in the OT of 10-dpc hearts could lead to reduced migration of NCCs into this structure, and in consequence to the OT septation defects persistent in this model (Yasui *et al.*, 1995; 1997; 1999; Nakajima *et al.*, 1996; 1997). As stated above, the gradient of CRABP II affected by RA at 11 dpc seems to be consistent with this hypothesis. However, further studies are required to determine whether in this case the abolished gradient of CRABP II reflects specifically the reduction in the number of migrating NCCs. Alternatively, both changes in spatial expression of CRABP II and RALDH2 may reflect the increased sensitivity of the affected heart areas to excess RA signaling.

Regardless of the specific mechanism it is evident that expression of both *Crabp I* and *II* as well as *Raldh2* genes (mRNA levels) is responsive to RA excess administered during the heart looping stage.

Based on our findings we cannot deduce what are the exact regulatory mechanisms of CRABPs' action in the RA signaling network, however, RA-induced regulation of mRNA and protein levels of both CRABPs at the stages of cardiac loop formation SHF cells addition to the outflow tract and subsequent OT alignment, suggests that these proteins may be involved in these major developing processes of the heart and may mediate RA signaling.

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